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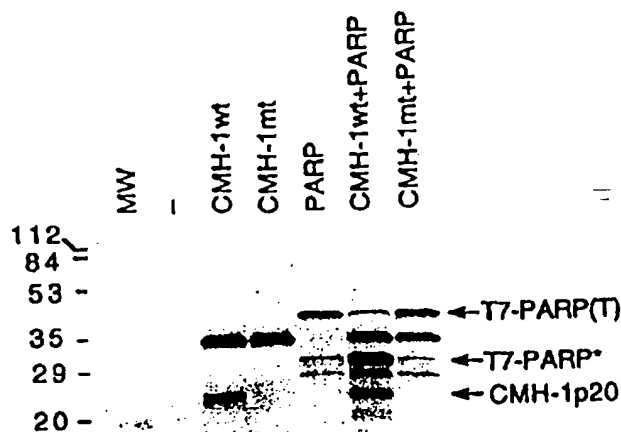
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/52, 9/64, C07K 16/40		A1	(11) International Publication Number: WO 97/16552 (43) International Publication Date: 9 May 1997 (09.05.97)
(21) International Application Number: PCT/US96/17431 (22) International Filing Date: 1 November 1996 (01.11.96) (30) Priority Data: 60/007,211 3 November 1995 (03.11.95) US 60/007,251 6 November 1995 (06.11.95) US 08/558,733 16 November 1995 (16.11.95) US (71) Applicant: VERTEX PHARMACEUTICALS INCORPORATED [US/US]; 130 Waverly Street, Cambridge, MA 02139-4242 (US). (72) Inventors: SU, Michael; 15 Donna Road, Newton, MA 02159 (US). LIPPKE, Judith, A.; 74 Knoll Street, Roslindale, MA 02131 (US). (74) Agents: HALEY, James, F., Jr. et al.; Fish & Neave, 1251 Avenue of the Americas, New York, NY 10020-1104 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG). Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM). European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	

(54) Title: DNA SEQUENCES ENCODING CMH-1, A CYSTEINE PROTEASE INVOLVED IN APOPTOSIS



(57) Abstract

The invention relates to DNA sequences encoding both active and inactive forms of CMH-1, a cysteine protease involved in programmed cell death. The invention further relates to the polypeptides encoded by those DNA sequences and antibodies to those polypeptides. The invention also relates to recombinant DNA molecules comprising these DNA sequences, as well as hosts transformed with such recombinant DNA molecules. The invention further relates to the use of the DNA sequences of this invention in gene therapy for both promoting and inhibiting apoptosis. The DNA sequences of this invention are also useful in diagnosing cells which have the potential to undergo apoptosis. Finally, the polypeptides encoded by the DNA sequences of this invention are useful in identifying inhibitors of apoptosis.

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DNA SEQUENCES ENCODING CMH-1, A CYSTEINE PROTEASE INVOLVED IN APOPTOSIS

TECHNICAL FIELD OF THE INVENTION

5 The invention relates to DNA sequences encoding active, acitvatable and inactive forms of CMH-1, a cysteine protease involved in programmed cell death. The invention further relates to the polypeptides encoded by those DNA sequences and antibodies to those polypeptides.

10 The invention also relates to recombinant DNA molecules comprising these DNA sequences, as well as hosts transformed with such recombinant DNA molecules. The invention further relates to the use of the DNA sequences of this invention in gene therapy for both promoting and

15 inhibiting apoptosis. The DNA sequences of this invention are also useful in diagnosing cells which have the potential to undergo apoptosis. Finally, the polypeptides encoded by the DNA sequences of this invention are useful in identifying inhibitors of

20 apoptosis.

BACKGROUND OF THE INVENTION

Programmed cell death (apoptosis) plays an important role in embryonic development, homeostasis, and in diseases such as neurodegenerative disorders,

25 autoimmune diseases and cancer [R. E. Ellis et al., Ann. Rev. Cell Biol., 7, pp. 663-98 (1991)]. Apoptosis is characterized by a set of cellular events including cell shrinkage, chromatin condensation, and DNA fragmentation. Activation of proteases is one of crucial events in the

30 cellular execution of apoptosis (S. J. Martin et al., Cell, 82, pp. 349-52 (1995)).

Genetic studies in the nematode *Caenorhabditis*

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C. elegans have provided evidence that *Ced-3* gene is indispensable for cell death during worm development [J. Yuan et al., Cell, 75, pp. 641-52 (1993)]. The CED-3 protein shares 28% sequence identity to mammalian interleukin-1 β (IL-1 β) converting enzyme (ICE) that cleaves the inactive IL-1 β precursor to the proinflammatory cytokine [N. A. Thornberry et al., Nature, 356, pp. 768-74 (1992)]. Both ICE and CED-3 are members of a newly-discovered cysteine protease family that also includes mammalian enzymes Tx/ICH-2/ICErelIII, ICErelIII, Nedd-2/ICH-1, CPP32/Yama/Apopain, and Mch2 [C. Faucheu et al., EMBO J., 9, pp. 1914-22 (1995); N. A. Munday et al., J. Biol. Chem., 270, pp. 15870-76 (1995); L. Wang et al., Cell, 78, pp. 739-50 (1994); S. Kumar et al., Genes Dev., 8, pp. 1613-26; T. Fernandes-Alnemri et al., J. Biol. Chem., 269, pp. 30761-64 (1994); T. Fernandes-Alnemri et al., Cancer Res., 55, pp. 2737-42 (1995)]. Each of these cysteine proteases are composed of two subunits of approximately 20 kDa and 10 kDa derived from processing of precursor polypeptides. They share conserved amino acid residues for catalysis and binding of P1 aspartate residue (K. P. Wilson et al., Nature, 370, pp. 270-73 (1994); and N. P. C. Walker et al., Cell, 78, pp. 343-52 (1994)].

Overexpression of cysteine proteases in the Ice/Ced-3 gene family leads to apoptosis of transfected fibroblast cells [M. Miura et al., Cell, 75, pp. 653-60 (1993); C. Faucheu et al., EMBO J., 9, pp. 1914-22 (1995); T. Fernandes-Alnemri et al., J. Biol. Chem., 269, pp. 30761-64 (1994); T. Fernandes-Alnemri et al., Cancer Res., 55, pp. 2737-42 (1995); Y. Gu et al., EMBO J., 9, pp. 1923-31 (1995); M. Tewari et al., Cell, 81, pp. 3255-60 (1995)], and ICE inhibitors block cell death of neurons deprived of neurotrophic factors [V. Gaglardini et al., Science, 263, pp. 826-28 (1994); and C. E.

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Milligan et al., Neuron, 15, pp. 385-93 (1995)]. The involvement of ICE or related proteases has been implicated in Fas- and TNF α -mediated apoptosis (K. Kuida et al., Science, 267, pp. 2000-03 (1995); M. Enari et al., Nature, 375, pp. 78-79 (1995); M. Los et al., Nature, 375, pp. 81-83 (1995); M. Tewari et al., J. Biol. Chem., 270, pp. 3255-3260 (1995); M. Miura et al., Cell, 75, pp. 653-660 (1995)].

Furthermore, ICE deficient mice are resistant to apoptosis induced by an anti-Fas antibody, suggesting a physiological role for ICE in Fas-mediated apoptosis of thymocytes (K. Kuida et al., 1995).

A number of proteins, including poly(ADP-ribose) polymerase (PARP), 70 kDa protein component of the U1 small nuclear ribonucleoprotein, lamin B1, β -actin and topoisomerase I are proteolytically degraded during apoptosis. Most notably, the rapid and complete cleavage of 116-kDa PARP between Asp214 and Gly215 to 31-kDa and 85-kDa polypeptides occurs at the onset of apoptosis [S. H. Kauffman et al., Cancer Res., 53, pp. 3976-85 (1993); Y. A. Lazebnik et al., Nature, 371, pp. 346-47 (1995)].

Some workers have suggested that CPP32 is the cysteine protease that cleaves PARP in a cytosolic extract of apoptotic cells [D. W. Nicholson et al., Nature, 376, pp. 37-43 (1995); Y. A. Lazebnik et al., (1995)]. Cytotoxic T lymphocyte (CTL)-specific granzyme B cleaves and activates CPP32, resulting in PARP cleavage in target cells, suggesting a protease activation cascade involving CPP32 in CTL-mediated apoptosis [A. J. Darmon et al., Nature, 377, pp. 446-48 (1995)]. In addition, CPP32 cleaves and activates sterol responsive element binding proteins (SREBP) that are involved in transcription regulation of genes for low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl CoA

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Synthase [X. Wang et al., J. Biol. Chem., 270, pp. 18044-50 (1995)].

Despite these developments to date, other members of the *Ice/Ced-3* gene family of cysteine proteases need to be identified and characterized in order to fully understand the mechanisms of apoptosis.

SUMMARY OF THE INVENTION

The invention provides a complete cDNA sequence encoding a 34-kDa polypeptide that is homologous to CPP32. The full-length cDNA encodes the proenzyme ("activatable") form of a cysteine protease -- CMH-1 -- that is important in programmed cell death ("apoptosis"). The invention also provides cDNA sequences encoding active forms of CMH-1, as well as inactive, mutated forms of CMH-1.

The invention also provides recombinant DNA molecules comprising any of the above-described cDNA sequences. The recombinant DNA molecules of this invention are used to transform both prokaryote and eukaryote hosts. Such transformed hosts are also part of this invention. The transformed hosts may be utilized to produce large quantities of the DNA sequences of this invention. Additionally, the transformed hosts of this invention may be utilized to produce any of the polypeptides encoded by those DNA sequences.

The DNA sequences of this invention may be used in both therapeutic and diagnostic applications. The DNA sequences encoding a pro-enzyme form or an active form of CMH-1 are suitable for use in gene therapy to promote apoptosis, especially in cancer cells. In addition, those DNA sequences may be utilized to modulate cholesterol levels in a patient, due to the ability of

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the expression product to activate elements involved in the transcription of low density lipoprotein receptors.

The DNA sequences encoding inactive, mutant forms of CMH-1 are useful in gene therapy to prevent apoptosis by competing with the native, active form of the enzyme in the cell. This therapeutic use is especially useful in preventing neurological diseases.

The DNA sequences of this invention may be used as diagnostic tools to detect and quantify Cmh-1 mRNA levels in various cells. This method may be used to determine the sensitivity of cancer cells to chemotherapeutic treatment. A high Cmh-1 mRNA level suggests that the cell would be responsive to chemotherapy. This method may also be used to detect diseases in cells that do not normally undergo apoptosis, especially cells involved in neurological disease. A high level of Cmh-1 mRNA would be indicative of a disease state.

The Cmh-1 DNA sequences of this invention may also be used to inhibit the expression of CMH-1 polypeptides in a cell through the use of anti-sense technology. Single stranded, antisense DNA can be introduced into cells where it can hybridize to and inhibit the translation of Cmh-1 mRNA. Such methods can be used to prevent undesirable apoptosis, such as is characteristic of neurological diseases.

The active CMH-1 polypeptides of this invention are useful as reagents for screening potential apoptosis inhibitors. The ability of a compound to block CMH-1-mediated cleavage of either its natural substrate, poly(ADP-ribose) polymerase ("PARP"), or an appropriate synthetic peptidyl substrate, suggests that such a compound is a potential apoptosis inhibitor.

The invention also provides monoclonal and polyclonal antibodies to CMH-1. These antibodies,

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may be used to assay for CMH-1 levels in a patient or cell. In addition, CMH-1 antibodies are useful in inhibiting CMH-1 activity, and therefore, apoptosis

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 depicts a comparison of the amino acid residues lining each of the substrate side-chain binding pockets of various members of the ICE cysteine protease family. Amino acid residues that are identical to those present in CMH-1 are shown in bold.

10 Figure 2, panel A, depicts an immunoblot of extracts of COS cells transfected with plasmids expressing T7-tagged derivatives of an activated form of CMH-1(Ala24-Gln303) (lane "CMH-1wt"), an inactivated mutant of CMH-1(Ala24-Gln303) (lane "CMH-1mt"), a
15 truncated PARP (Gu et al., 1995b) alone (lane "PARP"), truncated PARP and activated CMH-1(Ala24-Gln303) together (lane "CMH-1wt+PARP"), or truncated PARP and the CMH-1 mutant together (lane "CMH-1mt+PARP"). Immunoblotting was carried out using an anti-T7 antibody. Arrows
20 indicate the position of cleaved p20 CMH-1. "PARP(T)" denotes truncated PARP. "PARP(*)" denotes the tagged N-terminal fragment of cleaved PARP.

 Figure 2, panel B depicts the induction of apoptosis in transfected COS cells by active or inactive
25 CMH-1, as determined by internucleosomal DNA fragmentation.

 Figure 3 depicts the purification of recombinant (His)₆-tagged CMH-1 following Ni-affinity chromatography. Proteins were detected either by
30 Commassie blue staining (lane 1) or by immunoblotting with anti-(His)₆-tag antibody (lane 2).

 Figure 4, panel A depicts the ability of recombinant active CMH-1 to cleave PARP in the presence
~~or absence of the tetrapeptide aldehyde inhibitor~~

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DEVD-CHO (first four lanes). "PARP(T)" denotes the uncleaved truncated form of PARP. "PARP*" indicates the primary cleavage products (32 and 12 kDa).

Figure 4, panel A, also compares the ability of CMH-1 or ICE to cleave IL-1 β precursor (last three lanes). "pIL-1 β " denotes the uncleaved precursor. "IL-1 β " denotes the mature cleavage product.

Figure 4, panel B depicts the effects of various protease inhibitors on the ability of CMH-1 to cleave ³⁵S-labelled PARP in vitro, as analyzed by SDS-PAGE and fluorography.

Figure 5 depicts a Northern blot analysis of human Cmh-1 in heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and pancreas (lane 8) using full length CMH-1 cDNA as a probe.

DETAILED DESCRIPTION OF THE INVENTION

The following definitions are used throughout the specification and the claims.

"Cmh-1" refers to nucleotide sequences, either DNA or RNA, single-stranded or double-stranded that encode a CMH-1 polypeptide. When used to define a double-stranded nucleotide sequence, the term also refers to the anti-sense nucleotide sequence.

"CMH-1" and "CMH-1 polypeptide" refer to a polypeptide which shares at least 80%, and more preferably, at least 90% homology with amino acids 29-303 of SEQ ID NO:2.

An "active CMH-1 polypeptide" is a CMH-1 polypeptide that possesses cysteine protease activity and is capable of cleaving PARP by the assay set forth in Example 4. Examples of active CMH-1 polypeptides of this invention are amino acids 24-303 of SEQ ID NO: and amino

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acids 29-303 of SEQ ID NO:2.

An "activatable CMH-1 polypeptide" is an inactive form of a CMH-1 polypeptide which, upon cleavage, is converted into an active CMH-1 polypeptide (i.e., a proenzyme form). An example of an activatable CMH-1 polypeptide is SEQ ID NO:2.

An "inactive CMH-1 polypeptide" is a CMH-1 polypeptide that lacks cysteine protease activity, due to a mutation, deletion or substitution. Preferred examples of inactive CMH-1 polypeptides are those which have been mutated at one or more of the following catalytic residues: Cys186, Trp232 or Arg233 present in SEQ ID NO:2.

Additional terms are defined where necessary throughout the application.

According to one embodiment, the invention provides an isolated DNA sequence encoding an active, activatable or inactive CMH-1 polypeptide selected from SEQ ID NO:1; DNA sequences that hybridize to SEQ ID NO:1; and DNA sequences which but for the degeneracy of the genetic code would hybridize to SEQ ID NO:1 or sequences which hybridize thereto and code for a polypeptide having the same amino acid sequences coded for by SEQ ID NO:1 or sequences which hybridize thereto.

As used herein, the term "hybridize to" refers to the ability of a denatured DNA sequence to hydrogen bond to another denatured DNA sequence through complementary base pairs under conditions which allow sequences having at least 80% homology to form such hybrids. Such conditions are well known in the art and are exemplified by salt and temperature conditions substantially equivalent to 5X SSC and 65°C for both hybridization and wash.

Although only a single full-length cDNA sequence is set forth in the attached Sequence Listing,

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it will be readily apparent to those of skill in the art that any other DNA sequence which encode the same amino acid sequence (SEQ ID NO:2):

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M A D D Q G C I E E Q G V E D S A N E
5 D S V D A K P D R S S F V P S L F S K
  K K K N V T M R S I K T T R D R V P T
  Y Q Y N M N F E K L G K C I I I N N K
  N F D K V T G M G V R N G T D K D A E
  A L F K C F R S L G F D V I V Y N D C
10 S C A K M Q D L L K K A S E E D H T N
  A A C F A C I L L S H G E E N V I Y G
  K D G V T P I K D L T A H F R G D R C
  K T L L E K P K L F F I Q A C R G T E
  L D D G I Q A D S G P I N D T D A N P
15 R Y K I P V E A D F L F A Y S FT V P G
  Y Y S W R S P G R G S W F V Q A L C S
  I L E E H G K D L E I M Q I L T R V N
  D R V A R H F E S Q S D D P H F H E K
  K Q I P C V V S M L T K E L Y F S Q;

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20 are also be part of applicant's invention. Such DNA sequences merely utilize the redundancy of the genetic code to encode the same amino acids. While those specific sequences are not set forth herein due to space considerations, it should be understood that one of
 25 ordinary skill in the art could ascertain all of such DNA sequences merely by reference to the genetic code and without the exercise of inventive skill.

In addition to genetically redundant DNA sequences, the invention also includes DNA sequences
 30 which encode other amino acid sequences which are at least 80%, and preferably at least 90% homologous to SEQ ID NO:2. Because this aspect of the invention does not require the isolated DNA sequence to encode an active CMH-1, any nucleotides of SEQ ID NO:1 may be modified to
 35 produce a DNA sequence of this invention. The

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identification and isolation of these additional sequences may be achieved by standard DNA library screening techniques (hybridization, PCR) using SEQ ID NO:1 or portions thereof as a probe. Such homologous sequences may be found in any mammalian tissue cDNA library, as well as in insect cDNA libraries, yeast and other fungi cDNA libraries and prokaryotic cDNA libraries.

According to an alternate embodiment, the invention provides an isolated DNA sequence which encodes an active CMH-1 polypeptide selected from nucleotides 117-959 of SEQ ID NO:1, nucleotides 132-959 of SEQ ID NO:1; DNA sequences which hybridize to either of the former DNA sequences; and DNA sequences which but for the degeneracy of the genetic code would hybridize to and code for a polypeptide having the same amino acid sequence as any of the previous DNA sequences. Most preferably, the DNA sequences according to this embodiment are those that encode amino acids 24-303 or amino acids 29-303 of SEQ ID NO:2.

In this embodiment, the isolated DNA sequence must encode a catalytically active CMH-1. All DNA sequences which encode the same amino acids as nucleotides 117-959 of SEQ ID NO:1 (amino acids 24-303 of SEQ ID NO:2) or nucleotides 132-959 of SEQ ID NO:1 (amino acids 29-303 of SEQ ID NO:2) are clearly within the scope of this invention. Such genetically degenerate sequences can be deduced by those of ordinary skill in the art without the exercise of inventive skill utilizing the well-known genetic code.

Additional DNA sequences which encode active CMH-1 polypeptides may be identified by standard DNA library screening techniques using nucleotides 117-959 or 132-959 of SEQ ID NO:1 or portions thereof as a probe.

~~Such homologous DNA sequences should not contain~~

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nucleotides which alter the encoded catalytic residues His144 or Cys186 of SEQ ID NO:2. Preferably, such homologous DNA sequences will also not contain nucleotides which alter the P1 (Arg87, Gln184, Arg233, Ser239) site amino acids of SEQ ID NO:2. More preferred are homologues which do not contain nucleotides that alter the P2 (Tyr 229, Trp232), P3 (Asn 180, Pro 235) and P4 (Ser234, Arg237, Trp240, Ser275) site amino acids of SEQ ID NO:2. Even more preferred are homologous DNA sequences which contain nucleotides encoding only conservative amino acid substitutions for some or all of the other amino acids of SEQ ID NO:2. The translation products of any of these DNA sequences may then be assayed for cysteine protease activity as described below.

According to another embodiment, the invention provides an isolated DNA sequence encoding an inactive CMH-1 polypeptide selected from DNA sequences which hybridize to and are at least 80%, homologous to SEQ ID NO:1 and which encode an active site mutation at either amino acid 144 or 186 of SEQ ID NO:2; and DNA sequences which but for the degeneracy of the genetic code would hybridize to and code for a polypeptide having the same amino acid sequence as the former DNA sequences. More preferred are DNA sequences which encode mutations at both amino acid 144 and 186 of SEQ ID NO:1. Even more preferred are DNA sequences which additionally encode mutations at one or more of the following amino acids of SEQ ID NO:2: 87, 184, 233, 239, 229, 232, 180, 235, 234, 237, 240 and 275. Most preferred is a DNA sequence which encodes the following mutations in SEQ ID NO:2: Cys186-Ser, Trp232-Ala and Arg233-Glu.

As set forth above, DNA sequences according to this aspect of the invention may be identified and isolated through standard cDNA library screening.

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Specific mutations may be introduced into Cmh-1 DNA through the use of site-directed mutagenesis. The translation products of such DNA sequences may then be assayed for (lack of) cysteine protease activity as described below.

According to another embodiment of this invention, any of the DNA sequences described above may be employed in a recombinant DNA molecule. The isolated DNA sequences of this invention may be inserted into any of the numerous commercially or otherwise publicly available cloning vectors. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, e.g., E.coli plasmids col E1, pCR1, pBR322, PMB9 and their derivatives, plasmids such as RP4; phage DNAs, e.g., the numerous derivatives of phage λ , e.g., NM989, and other phage DNA, e.g., M13 and Filamentous single stranded phage DNA; yeast plasmids such as the 2 μ plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

The choice of cloning vector will, of course, depend upon the cell type(s) to be transformed with the resulting recombinant DNA molecule, as well as the ultimate use of that recombinant DNA molecules (i.e., DNA production, gene therapy, polypeptide expression). Preferred eukaryotic vectors are SV40-derived vectors. Preferred prokaryotic vectors are *E. coli* expression vectors. Preferred viral vectors are modified eukaryotic viral vectors, preferably attenuated adenovirus vectors, that may be used in gene therapy, such as those described

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in PCT publications WO 94/26915 and WO 94/28938, the disclosures of which are herein incorporated by reference.

Techniques for inserting a DNA sequence of this invention into a vector to produce a recombinant DNA molecule involve standard molecular biological techniques and are well known in the art (see, for example, J. Sambrook, et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989)).

According to a preferred embodiment, the recombinant DNA molecule of this invention will additionally comprise an expression control sequence operatively linked to a DNA sequence of this invention. The term "expression control sequence" refers to a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. It includes both 5' and 3' non-coding DNA sequencing and optionally includes an ATG start codon.

The term "operatively linked" refers to the positioning of an expression control sequence with respect to a coding DNA sequence of interest such that the expression control sequence controls and regulates the transcription and translation of that DNA sequence. The term "operatively linked" includes having an appropriate start signal (e.g., ATG) in front of the DNA sequence to be expressed and maintaining the correct reading frame to permit expression of the DNA sequence under the control of the expression control sequence and production of the desired product encoded by the DNA sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal, such a start signal can be inserted in front of the gene.

The choice of expression control sequence

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depends upon the nature of the recombinant DNA molecule, the host that will be transformed by that recombinant DNA molecule, and whether constitutive or inducible expression of a DNA sequence of this invention is
5 desired. Such useful expression control sequences include, for example, the early and late promoters of SV40 or adenovirus, the lac system, the trp system, the TAC or TRC system, the major operator and promoter regions of phage λ , the control regions of fd coat
10 protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase (e.g., Pho5), the promoters of the yeast α -mating factors, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or
15 their viruses, and various combinations thereof.

These recombinant DNA molecules are used to produce CMH-1 polypeptides. Preferred expression control sequences are the SR α promoter, when a eukaryotic vector is employed, and any inducible promoter when a
20 prokaryotic vector is employed. Most preferred are the expression control sequences contained in the commercially available pET-15b *E. coli* vector (Novagen).

The invention also provides host cells transformed by the recombinant DNA molecules of this
25 invention. These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of E.coli, Pseudomonas, Bacillus, Streptomyces, fungi such as yeasts, and animal cells, such as CHO, R1.1, B-W and L-M cells, African Green Monkey kidney cells (e.g., COS 1,
30 COS 7, BSC1, BSC40, and BMT10), insect cells (e.g., Sf9), and human cells and plant cells in tissue culture. Eukaryotic cells may harbor the recombinant DNA molecules of this invention as an extrachromosomal element or incorporate all or part of it into the host chromosome.

35 It will be understood that not all vectors,

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expression control sequences and hosts will function equally well to replicate and/or express the DNA sequences of this invention. Neither will all hosts function equally well with the same expression system.

5 However, one skilled in the art will be able to select the proper vectors, expression control sequences, and hosts without undue experimentation to accomplish the desired expression without departing from the scope of this invention. For example, in selecting a vector, the
10 host must be considered because the vector must function in it. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic and other selective markers, will also be considered.

15 In selecting an expression control sequence, a variety of factors will normally be considered. These include, for example, the relative strength of the system, its controllability, and its compatibility with the particular DNA sequence or gene to be expressed,
20 particularly as regards potential secondary structures. Suitable unicellular hosts will be selected by consideration of, e.g., their compatibility with the chosen vector, their ability to fold proteins correctly, and their fermentation requirements, as well as the
25 toxicity to the host of the product encoded by the DNA sequences to be expressed, and the ease of purification of the expression products.

Considering these and other factors a person skilled in the art will be able to construct a variety of
30 vector/expression control sequence/host combinations that will replicate and/or express the DNA sequences of this invention on fermentation or in large scale animal culture.

Methods for transforming cells with recombinant
35 DNA molecules are well known in the art (see, for

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example, J. Sambrook, et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989)). Any of those methods may be employed to produce the transformed hosts of this invention. Identification of transformed hosts may be achieved by assaying for the presence of Cmh-1 DNA, Cmh-1 RNA or a CMH-1 polypeptide. Additionally, transformants may be identified by growth in selective media. For this assay, the gene necessary for growth in selective media is cotransfected into the cell either on the same or a different recombinant DNA molecule as the Cmh-1 DNA and is expressible in that cell. It will, of course, be obvious that the gene for selective growth should not present in the untransformed cell.

The transformed hosts of this invention may be employed to produce either large quantities of Cmh-1 DNA sequences of this invention and/or a CMH-1 polypeptide encoded thereby. In order to produce large quantities of Cmh-1 DNA, the host, preferably a prokaryotic host, is grown in a medium and under conditions that promote DNA replication and cell division. Any complete media routinely used to grow bacteria is suitable for this purpose. Following growth, the transformed cells are separated from the growth medium and plasmid DNA is then isolated from the cells by standard and well-known techniques. Cmh-1 DNA may then be excised from the plasmid through the use of restriction endonucleases.

When the transformed hosts of this are employed to produce a CMH-1 polypeptide, it is preferred that the CMH-1 polypeptide be active or activatable. In this embodiment, the preferred host is a mammalian cell. The transformed host should be grown in a medium that promotes expression of the CMH-1 polypeptide-encoding DNA sequence present in that host. If expression of that DNA sequence is under the control of a constitutive promoter,

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any standard growth medium is suitable. If the CMH-1 DNA is under the control of an inducible promoter, the growth medium should be supplemented with a compound that induces expression or growth conditions should be altered so as to induce expression (i.e., change in growth temperature). Following expression, the transformed cells are separated from the growth medium, lysed and the CMH-1 polypeptide purified by standard methods.

In order to determine the presence of an active CMH-1 polypeptide, a protease activity assay is used. Such an assay measures the ability of the polypeptide to cleave natural or synthetic substrates. Active CMH-1 polypeptides cleave Asp-X bonds. The preferred natural substrate for these assays is poly(ADP-ribose) polymerase ("PARP"). Synthetic substrates useful in these assays are peptides containing an Asp-X bond. A preferred assay for detecting active CMH-1 polypeptide is described in Example 4.

In addition to the CMH-1 polypeptides, the invention also provides antibodies to CMH-1 polypeptides. The generation of antibodies may be achieved by standard methods in the art for producing both polyclonal and monoclonal antibodies using a CMH-1 polypeptide or fragment thereof as antigen. Such antibodies or active fragments thereof (such as Fab fragments), may be used to assay *in vivo* or *in vitro* levels of CMH-1 polypeptide. Increased *in vivo* levels of CMH-1 polypeptides may be indicative of the onset of apoptosis.

Antibodies to CMH-1 polypeptides also have potential therapeutic use in preventing apoptosis in a patient. Alternatively, the CMH-1 antibodies may be used to regulate cholesterol levels in a patient. As stated previously, the cysteine proteases involved in apoptosis also activate sterol responsive element binding proteins, which in turn regulate transcription of genes encoding

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the LDL receptor and 3-hydroxy-3-methylglutaryl CoA Synthase (X. Wang et al., (1995)).

Such antibodies may be directed to their target cells through conjugation to cell-specific molecules.

5 The conjugation of such antibodies and the identity of cell-specific targeting molecules is known in the art.

Finally, CMH-1 polypeptide antibodies may be employed to purify CMH-1 polypeptides from either native sources or transformed hosts expressing CMH-1
10 polypeptides. For example, the antibodies can be conjugated by standard techniques to an insoluble matrix to form an immunoaffinity resin.

The Cmh-1 DNA sequences of this invention, of fragments thereof, may be used to identify homologous DNA
15 sequences so as to potentially identify other members of this cysteine protease family. These DNA sequences, or fragments thereof, The DNA sequences of this invention are useful in a number of therapeutic and diagnostic applications.

20 According to one embodiment, the DNA sequences encoding an active or activatable CMH-1 polypeptide may be used in either *in vitro* or *in vivo* gene therapy. In this embodiment, the DNA must be contained in a suitable vehicle for gene therapy. Such vehicles are known in the
25 art. For example, various viruses that are capable of transferring genetic material to a target cell, without replicating in that cell have been described (United States patents 5,112,767, 5,240,846 and 5,112,767, the disclosures of which are herein incorporated by
30 reference). Such viruses include replication-defective adenoviruses, adeno-associated viruses (AAV), and replication defective retroviruses, such as PLJ, pZip, pWe and pEM.

Gene delivery systems other than viruses can
35 also be employed in the methods of this invention. For

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example, the gene to be transferred may be packaged in a liposome. When cells are incubated with DNA-encapsidated liposomes, they take up the DNA and express it. To form these liposomes, one mixes the DNA of an expression
5 vector which expresses the gene to be transferred with lipid, such as N-[1-(2,3,dioleoyloxy)propyl]-N,N,N-trimethylammonium-chloride (DOTMA) in a suitable buffer, such as Hepes buffered saline. This causes the spontaneous formation of lipid-DNA complexes (liposomes)
10 which can be employed in the methods of this invention [P. L. Felgner et al., Proc. Natl. Acad. Sci. USA, 84, pp. 7413-17 (1987)].

Another gene delivery system that may be utilized in this invention is DNA-protein complexes. The
15 ~~formation~~ formation of these complexes is described in United States patent 5,166,320, the disclosure of which is herein incorporated by reference. Specifically, these complexes comprise the gene to be transferred (together with promoter, enhancer sequences and other DNA necessary
20 for expression in the target cell) linked via a suitable polymer, such as polylysine, to a polypeptide ligand for a receptor or other cell surface protein. This complex is taken up by the target cells via endocytosis after the ligand binds to the cell surface receptor. The DNA is
25 then cleaved from the rest of the complex via intracellular enzymes which cut the polymer linker. Such complexes are particularly useful in targeting specific cell types. For example, cancer cells may be
30 specifically targeted through the use of proteins which specifically recognize tumor cell surface markers or receptors.

The preferred use according to this embodiment is in the treatment of cancer in a patient. The term "patient", as used herein refers to any mammal,
35 especially humans. Cancer cells transformed with a DNA

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sequence encoding an active or activatable CMH-1 polypeptide will express that DNA, which in turn, will cause cell death.

DNA sequences encoding inactive CMH-1 polypeptides are also useful in gene therapy to inhibit apoptosis. According to this embodiment, the inactive CMH-1 polypeptide encoded by this DNA would competitively compete for substrate with native, active CMH-1. This would decrease the CMH-1 activity in the cell, inhibiting apoptosis. Preferred cells to be treated by this method are nerve cells.

The DNA sequences of this invention may also be used as anti-sense inhibitors of CMH-1 polypeptide mRNA and thus inhibitors of apoptosis. In this embodiment, single stranded, anti-sense Cmh-1 DNA is introduced into cells by standard techniques. Once inside the cell, the DNA binds to Cmh-1 mRNA and prevents it from being translated.

According to an alternate embodiment, the DNA sequences of this invention may be used as a diagnostic tool to determine Cmh-1 mRNA levels in cells. This may be achieved by standard Northern blot or dot blotting techniques that are well known in the art. For example, a low level of Cmh-1 mRNA in cancer cells is indicative that those cells will respond to chemotherapy. In normal cells, high levels of Cmh-1 mRNA may be predictive of disease, especially in nerve and other neurological cells.

The active or activatable CMH-1 polypeptides of this invention are useful assay reagents for identifying and characterizing potential apoptosis inhibitors. In this embodiment, potential inhibitors are added to an assay mixture containing an active or activatable CMH-1 polypeptide and a substrate. If an activatable form is used, the assay must employ conditions which convert that

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form to an active form. Preferably, an active form of CMH-1 polypeptide is used. Inhibition of a CMH-1 polypeptide's ability to cleave its substrate by a compound suggest that that compound is a potential inhibitor of apoptosis.

In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only and are not to be construed as limiting this invention in any manner.

EXAMPLE 1

Cloning of CMH-1 cDNA

We initially screened the GenBank EST database maintained by the National Center for Biotechnology Information (NCBI) Sequence for cDNA clones encoding ICE-like polypeptides using the Blast algorithm [S. F. Altshul et al., J. Mol. Biol., pp. 403-10 (1990)]. We generated initial multiple sequence alignments of known ICE family protein peptide sequences using the MACAW program (G. D. Schuler et al., Proteins: Structure, Function, and Genetics, 9, pp. 180-90 (1991)) and later refined by the combination of the PILEUP algorithm (Genetics Computer Group, 1995) and pairwise comparison.

Through this screen, we identified a partial human cDNA clone deposited by the Washington University-Merck EST project (accession number T50828) which had a partial open reading frame with limited similarity to our consensus ICE sequence and a 3' untranslated region. We obtained the partial human cDNA clone 72778 corresponding to this EST (expressed sequence tag) sequence through the IMAGE Consortium (Lawrence Livermore National Laboratory).

In order to generate a full-length cDNA clone, we ligated a spleen cDNA pool with a DNA adaptor

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(Clontech, Palo Alto, CA) and subsequently performed PCR using a primer to the 5' adaptor (SEQ ID NO:3:

5'-CCATCCTAATACGACTCACTATAGGGC and a primer to the EST T50828 sequence SEQ ID NO:4: (5'-GCAAACCTCTGTCAATTCACCC).

5 Through this technique, we were able to generate a 0.8 kb SacI-HgiAI PCR fragment containing the 5' coding region. We then ligated this fragment to the 1.55 kb HgiAI-KpnI fragment from the 72778 clone containing the 3' region subcloned the resulting DNA into pBluescript SK
10 (Stratagene, La Jolla CA) at SacI/KpnI to generate the full length cDNA.

We sequenced the resulting cDNA, named Cmh-1 (CPP32/Mch2 homolog-1), on both strands by ABI Prism Dye Deoxy Terminator sequencing using the ABI 373 DNA
15 Sequencer. The CMH-1 sequence is set forth in the Sequence Listing as SEQ ID NO:1.

Analysis of the full length cDNA sequence identified a single open reading frame of 303 amino acids encoding a protein of 34 kDa, which we have named CMH-1
20 The encoded protein is set forth in the Sequence Listing as SEQ ID NO:2. We identified the catalytic residues as His144 and Cys186 in SEQ ID NO:2.

CMH-1 was found to have 52% identity to CPP32 identified by T. Fernandes-Alnemri et al. (1994). In
25 addition, there was sequence similarity with all members of the ICE/Ced3 gene family. Overall, the polypeptide can be divided into three domains, roughly corresponding to the P15, P20, and P10 subdomains of ICE. In CMH-1 these domains are 2, 21, and 11 kD, respectively.

30 There is strong similarity between CMH-1 and the sequence that comprises the core of the ICE structure, as well as a number of residues surrounding the active site Cys285 and His237 in the ICE crystal structure [K. P. Wilson et al., Nature, (1994); N. P. C.
35 Walker et al., Cell, (1994)]. Superposition of the CMH-1

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sequence onto this ICE structure allowed the identification of a number of other residues located around the protease active site (Fig. 1).

Interestingly, all of the residues which line the substrate binding pocket at the P1 position are identical with those found in ICE, including Arg87 and Arg233, corresponding to ICE Arg179 and Arg341. This strongly suggests that CMH-1 has substrate specificity for Asp in this position, as seen in other members of this family.

There is much less sequence conservation in the binding pockets corresponding to the P3-P4 residues between CMH-1 and its closest homolog CPP32, suggesting that CMH-1 has a different substrate(s).

EXAMPLE 2

Expression And Processing of CMH-1 in COS cells

We used transient expression of CMH-1 in COS cells to determine if CMH-1 has the predicted cysteine protease activity. Removal of the pro-domain from the CPP32 precursor occurs between Asp28 and Ser29 [D. W. Nicholson et al., Nature, (1995)]. For CMH-1, we estimated the position at which the prodomain removal might occur as Asp23-Ala24.

We therefore produced a cDNA fragment encoding an N-terminally T7-tagged CMH-1 lacking the first 23 amino acid residues by PCR using the following primers: SEQ ID NO:5: 5'-GCTGCAGTCTAGAGCTCCATGGCTAGCATGACTGGTG GACAGCAAATGGGTGCTAAGCCAGACCGGTCCTCG-3' and SEQ ID NO:6: 5'-GAATTCTAGATTCTATTGACTGAAGTAGAGTTC-3'. We digested the resulting amplified DNA with XbaI and subcloned it into an XbaI-cut SV40-based expression vector under the control of SR α promoter (pcDLSR α) [Y. Takebe et al., Mol. Cell. Biol., 8, pp. 466-72 (1988)].

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We produced a corresponding inactive mutant CMH-1 containing a Cys186→Ser, a Trp232→Ala and a Arg233→Glu mutation (C186S/W232A/R233E) by site-directed mutagenesis using oligonucleotides SEQ ID NO:7:

5 5'-CATTCAGGCTAGCCGAGGGAC and SEQ ID NO:8:

5'-GCTATTACTCAGCTGAGAGCCCAGGA and following well-known techniques [T. A. Kunkel, Proc. Natl. Acad. Sci. USA, 82, pp. 488-92 (1985); Y. Gu et al., EMBO J., (1995), the disclosures of which are herein incorporated by
10 reference]. These substitutions were at the conserved residues for catalysis and P1 aspartate binding.

We transiently transfected COS cells using the DEAE-dextran method as described by Y. Gu et al., EMBO J., (1995). COS cells were grown in 6 well culture
15 plates and transfected with 3μg of plasmid DNA containing -with the active or inactive CMH-1 coding sequences. We harvested the cells 24 hours after transfection.

Transfection of the expression plasmid in COS cells results in high expression and processing of the
20 truncated CMH-1 protein. Immunoblotting of the COS cell lysates with an anti-T7 antibody detected the unprocessed precursor with an apparent molecular weight of 34 kDa and a processed product with an apparent molecular weight of 22 kDa (Fig. 2, panel A).

25 When we co-expressed T7-CMH-1 with a 43 kDa truncated form of T7-tagged PARP, the PARP was cleaved into a T7-tagged 31-kDa fragment, similar to the cleavage of PARP by CPP32, ICE, Tx, and Nedd-2 [Y. Gu et al., J. Biol. Chem., 270, pp. 18715-18 (1995)]. The inactive
30 mutant (C186S/W232A/R233E) was defective in both autoprocessing and PARP cleavage.

Similar to other ICE-like cysteine proteases, we found that overexpression of the CMH-1 protein induces
35 shrinkage and DNA fragmentation in the transfected cells.

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(Fig. 2, panel B). It was apparent that induction of apoptosis was dependent on the presence of cysteine protease activity because expression of the active site mutant did not result in apoptosis of transfected COS cells (Fig. 2, panel B).

EXAMPLE 3

Expression and Purification of Recombinant CMH-1 In E. coli

To further characterize the activity of CMH-1 protein, we expressed an N-terminal (His)₆-tagged CMH-1(Ala24-Gln303) in *E. coli*. We constructed an expression plasmid for the N-terminal (His)₆-tagged CMH-1(Ala24-Gln303) by introducing in-frame XhoI sites to 5' and 3' ends of *Cmh-1* cDNA using PCR with the following primers:

SEQ ID NO:9: 5' CGGCTCGAGGCTAAGCCAGACCGGTCCTCG and
SEQ ID NO:10: 5' GGGCTCGAGCTATTGACTGAAGTAGAGTTC. The resulting XhoI fragment was ligated into an XhoI-cut pET-15b inducible *E. coli* expression vector (Novagen, Madison, WI). The resulting plasmid encodes a polypeptide of 303 amino acids with the 23-residue peptide SEQ ID NO:11: (MGSSHHHHHSSGLVPRGSHMLE, where LVPRGS (amino acids 14-19 of SEQ ID NO:11) represents a thrombin cleavage site fused in frame to the N-terminus of CMH-1 starting at Ala24, as confirmed by DNA sequencing as well as by N-terminal sequencing of the expressed proteins.

E. coli strain BL21(DE3) carrying this plasmid was induced with 0.8 mM IPTG, harvested and lysed in a microfluidizor in a buffer containing 20 mM sodium phosphate (pH 6.8), 300 mM NaCl, 2 mM DTT, 10% glycerol, 0.4 mM PMSF, 2.5 µg/ml leupeptin. We cleared the lysates by centrifugation at 100,000 x g for 30 minutes.

The supernatant containing soluble CMH-1 were .

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loaded onto a 0.5-ml nickel-NTA column (Qiagen) and washed with the same buffer. The column was then eluted with increasing concentrations of imidazole in the same buffer, and CMH-1 protein was eluted at 100-200 mM to
5 give a yield of approximately 5 mg of protein per liter of culture. Imidazole was then removed by dialysis.

The purified protein fraction contained three major polypeptides of approximately 34 kDa, 22 kDa, and 12 kDa (Figure 3). An 11 kDa polypeptide was also present in
10 some preparations.

Immunoblotting of the *E. coli* expressed (His)₆-tagged CMH-1 protein with an anti-(His)₆-tag antibody were carried out as described by Gu et al., J. Biol. Chem. (1995). The N-terminus of each of these
15 peptides was analyzed using the ABI 477A Protein Sequencer (Applied Biosystems, Inc., Foster City, CA).

These studies indicated that the 34 kDa and 22 kDa polypeptides contain the (His)₆-tag and the predicated N-terminal sequence of the truncated CMH-1
20 starting at Ala24. The N-terminus of 12 kDa and 11 kDa polypeptides start at Ser199 and Ala207, respectively, both of which are preceded by an Asp residue, suggesting that autoprocessing of the CMH-1 (Ala24-Gln303) protein occurs predominantly at the Asp198-Ser199 site and to a
25 lesser extent at the Asp206-Ala207 site. The 22 kDa and 12 kDa proteins are the autoprocessing products of the full length 34 kDa protein. They represent the two subunits of the CMH-1 protease.

EXAMPLE 4

In vitro cleavage of PARP by CMH-1

30

In order to determine the activity of purified CMH-1, we determined whether the protein could cleave ³⁵[S]-labeled pro-IL-1b and PARP and compared its cleavage activity to purified ICE. We prepared ³⁵[S]-labeled PARP

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and IL-1b precursor (pro-IL-1b) by in vitro transcription and translation. Cleavage experiments were carried out as described previously [Y. Gu et al., J. Biol. Chem. (1995)]. Cleavage products were analyzed by SDS-PAGE and
5 fluorography as described in that reference.

The results demonstrated that CMH-1 cleaved PARP but not pro-IL-1b. Less than 3 nM CMH-1 was required to completely process PARP within 30 minutes (Fig. 4, panel A). This efficiency was significantly
10 higher than that of ICE in cleaving the same substrate under identical conditions, and was similar to the activity of CPP32 against PARP (results not shown) or ICE against pro-IL-1b [Y. Gu et al., J. Biol. Chem., (1995)].

The cleavage activity of CMH-1 was inhibited by
15 the tetrapeptide inhibitor DEVD-CHO [D. W. Nicholson et al., Nature, (1995); X. Wang et al., J. Biol. Chem. (1995)], as well as by the cysteine-alkylating reagents N-ethylmaleimide and iodoacetamide, but not by the ICE inhibitors YVAD-CHO [N. A. Thornberry et al., Nature,
20 (1993); Y. Gu et al., J. Biol. Chem. (1995)] or crmA (a viral protein that is a specific ICE inhibitor) or inhibitors of serine proteases or metalloproteases (Fig. 4, panel B). These results demonstrate that CMH-1 is a cysteine protease with properties closer to CPP32 than to
25 ICE. This was consistent with the relative sequence identity between CPP32 and CMH-1.

EXAMPLE 5

Northern blot analysis of CMH-1 mRNA

We investigated the expression of Cmh-1 mRNA in
30 adult HUMAN tissues was investigated by the Northern blotting using a Cmh-1 specific cDNA probe. A human multiple tissue RNA blot (MTN blot, Clontech) containing 2µg/lane of poly (A)⁺ RNA was hybridized to the ³²[P]-labeled probe of full length Cmh-1 cDNA in 50%

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formamide, 10x Denhardts, 2% SDS, 5x SSPE and 100 µg/ml sheared and denatured ssDNA at 42°C for 16hrs. The blot was washed in 2x SSC, 0.1% SDS at room temperature for 40 minutes and at 0.1x SSC, 0.1% SDS at 42°C for 20 minutes and exposed to Kodak XAR-5 film for 18 hours.

5 A 2.5 kb mRNA was detected in tissues including pancreas, lung, placenta, kidney, muscle and heart. Notably, the expression of Cmh-1 was low in liver and almost undetectable in brain (Figure 5). This ubiquitous expression of Cmh-1 in a variety of tissues is similar to that of CPP32 (data not shown).

10 While I have hereinbefore presented a number of embodiments of this invention, it is apparent that my basic construction can be altered to provide other
15 embodiments which utilize the methods of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the claims appended hereto rather than the specific embodiments which have been presented hereinbefore by way of example.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Vertex Pharmaceuticals Incorporated
- (B) STREET: 130 Waverly Street
- (C) CITY: Cambridge
- (D) STATE: Massachusetts
- (E) COUNTRY: United States of America
- (F) POSTAL CODE (ZIP): 02139-4242
- (G) TELEPHONE: 617-577-6000
- (H) TELEFAX: 617-577-6680

(ii) TITLE OF INVENTION: DNA ENCODING CMH-1 - A CYSTEINE PROTEASE INVOLVED IN APOPTOSIS

(iii) NUMBER OF SEQUENCES: 11

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 60/007,211
- (B) FILING DATE: 03-NOV-1995

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 60/007,251
- (B) FILING DATE: 06-NOV-1995

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/558,733
- (B) FILING DATE: 16-NOV-1995

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2341 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
 - (B) LOCATION: 54..962
 - (D) OTHER INFORMATION: /function= "cysteine protease"
- /product= "CMH-1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

-30-

CTAATGCGGA GAGAGACTGT GCCAGTCCCA GCCGCCCTAC CGCCGTGGGA ACG ATG	56
Met	
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GCA GAT GAT CAG GGC TGT ATT GAG GAG CAG GGG GTT GAG GAT TCA GCA	104
Ala Asp Asp Gln Gly Cys Ile Glu Glu Gln Gly Val Glu Asp Ser Ala	
5 10 15	
AAT GAA GAT TCA GTG GAT GCT AAG CCA GAC CGG TCC TCG TTT GTA CCG	152
Asn Glu Asp Ser Val Asp Ala Lys Pro Asp Arg Ser Ser Phe Val Pro	
20 25 30	
TCC CTC TTC AGT AAG AAG AAG AAA AAT GTC ACC ATG CGA TCC ATC AAG	200
Ser Leu Phe Ser Lys Lys Lys Asn Val Thr Met Arg Ser Ile Lys	
35 40 45	
ACC ACC CGG GAC CGA GTG CCT ACA TAT CAG TAC AAC ATG AAT TTT GAA	248
Thr Thr Arg Asp Arg Val Pro Thr Tyr Gln Tyr Asn Met Asn Phe Glu	
50 55 60 65	
AAG CTG GGC AAA TGC ATC ATA ATA AAC AAC AAG AAC TTT GAT AAA GTG	296
Lys Leu Gly Lys Cys Ile Ile Ile Asn Asn Lys Asn Phe Asp Lys Val	
70 75 80	
ACA GGT ATG GGC GTT CGA AAC GGA ACA GAC AAA GAT GCC GAG GCG CTC	344
Thr Gly Met Gly Val Arg Asn Gly Thr Asp Lys Asp Ala Glu Ala Leu	
85 90 95	
TTC AAG TGC TTC CGA AGC CTG GGT TTT GAC GTG ATT GTC TAT AAT GAC	392
Phe Lys Cys Phe Arg Ser Leu Gly Phe Asp Val Ile Val Tyr Asn Asp	
100 105 110	
TGC TCT TGT GCC AAG ATG CAA GAT CTG CTT AAA AAA GCT TCT GAA GAG	440
Cys Ser Cys Ala Lys Met Gln Asp Leu Leu Lys Lys Ala Ser Glu Glu	
115 120 125	
GAC CAT ACA AAT GCC GCC TGC TTC GCC TGC ATC CTC TTA AGC CAT GGA	488
Asp His Thr Asn Ala Ala Cys Phe Ala Cys Ile Leu Leu Ser His Gly	
130 135 140 145	
GAA GAA AAT GTA ATT TAT GGG AAA GAT GGT GTC ACA CCA ATA AAG GAT	536
Glu Glu Asn Val Ile Tyr Gly Lys Asp Gly Val Thr Pro Ile Lys Asp	
150 155 160	
TTG ACA GCC CAC TTT AGG GGG GAT AGA TGC AAA ACC CTT TTA GAG AAA	584
Leu Thr Ala His Phe Arg Gly Asp Arg Cys Lys Thr Leu Leu Glu Lys	
165 170 175	
CCC AAA CTC TTC TTC ATT CAG GCT TGC CGA GGG ACC GAG CTT GAT GAT	632
Pro Lys Leu Phe Phe Ile Gln Ala Cys Arg Gly Thr Glu Leu Asp Asp	
180 185 190	
GGC ATC CAG GCC GAC TCG GGG CCC ATC AAT GAC ACA GAT GCT AAT CCT	680
Gly Ile Gln Ala Asp Ser Gly Pro Ile Asn Asp Thr Asp Ala Asn Pro	
195 200 205	
CGA TAC AAG ATC CCA GTG GAA GCT GAC TTC CTC TTC GCC TAT TCC ACG	728
Arg Tyr Lys Ile Pro Val Glu Ala Asp Phe Leu Phe Ala Tyr Ser Thr	
210 215 220 225	
GTT CCA GGC TAT TAC TCG TGG AGG AGC CCA GGA AGA GGC TCC TGG TTT	776
Val Pro Gly Tyr Tyr Ser Trp Arg Ser Pro Gly Arg Gly Ser Trp Phe	

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230	235	240	
GTG CAA GCC CTC TGC TCC ATC CTG GAG GAG CAC GGA AAA GAC CTG GAA 824			
Val Gln Ala Leu Cys Ser Ile Leu Glu Glu His Gly Lys Asp Leu Glu			
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ATC ATG CAG ATC CTC ACC AGG GTG AAT GAC AGA GTT GCC AGG CAC TTT 872			
Ile Met Gln Ile Leu Thr Arg Val Asn Asp Arg Val Ala Arg His Phe			
260	265	270	
GAG TCT CAG TCT GAT GAC CCA CAC TTC CAT GAG AAG AAG CAG ATC CCC 920			
Glu Ser Gln Ser Asp Asp Pro His Phe His Glu Lys Lys Gln Ile Pro			
275	280	285	
TGT GTG GTC TCC ATG CTC ACC AAG GAA CTC TAC TTC AGT CAA 962			
Cys Val Val Ser Met Leu Thr Lys Glu Leu Tyr Phe Ser Gln			
290	295	300	

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 TTTTATG CTCTTGAAAT ATTCAGAAAT TCTCCAGGAT TTTAATTTCA GGAAATGTA 1082
 TTGATTCAAC AGGGAAGAAA CTTTCTGGTG CTGTCTTTTG TTCTCTGAAT TTTAGAGAC 1142
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 TTTGTATGTC TGTTACCTTG TTAATAGACT TAATACATGC AACAGAAGTG ACTTCTGGAG 1262
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 TGGCAAAAAG AATCCCAATG TTTGACAAA CACAGCCAAG GGGATATTTA CTGCTCTTTA 1382
 TTGCAGAATG TGGGTATTGA GTGTGATTG AATGATTTTT CATTGGCTTA GGGCAGATTT 1442
 TCATGCAAAA GTTCTCATAT GAGTTAGAGG AGAAAAAGCT TAATGATTCT GATATGTATC 1502
 CATCAGGATC CAGTCTGGAA AACAGAAACC ATTCTAGGTG TTTCAACAGA GGGAGTTTAA 1562
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 ACAGATAAGA GAATACCTTG GTCTCACCTA TCCTGCCCTC ACATCTTCCA CCAGCACCTT 1862
 ACTGCCCAGG CCTATCTGGA AGCCACCTCA CCAAGGACCT TGGAAGAGCA AGGGACAGTG 1922
 AGGCAGGAGA AGAACAAGAA ATGGATGTAA GCCTGGCCCA TAATGTGAAC ATAAGTAATC 1982
 ACTAATGCTC AACAATTTAT CCATTCAATC ATTTATTCAT TGGGTTGTCA GATAGTCTAT 2042
 GTATGTGTAA AACAATCTGT TTTGGCTTTA TGTGCAAAAT CTGTTATAGC TTTAAATAT 2102
 ATCTGGAAT TTTTAGATTA TTCCAAGCCT TATTTTGAGT AAATATTTGT TACTTTTAGT 2162
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AACAAATGTT CATATTTATT GATTAAAAAT GTGGTTGCTT AAAAAAAAAA AAAAAAAAAA 2341

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 303 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 Pro Ser Leu Phe Ser Lys Lys Lys Lys Asn Val Thr Met Arg Ser Ile
 35 40 45
 Lys Thr Thr Arg Asp Arg Val Pro Thr Tyr Gln Tyr Asn Met Asn Phe
 50 55 60
 Glu Lys Leu Gly Lys Cys Ile Ile Ile Asn Asn Lys Asn Phe Asp Lys
 65 70 75 80
 Val Thr Gly Met Gly Val Arg Asn Gly Thr Asp Lys Asp Ala Glu Ala
 85 90 95
 Leu Phe Lys Cys Phe Arg Ser Leu Gly Phe Asp Val Ile Val Tyr Asn
 100 105 110
 Asp Cys Ser Cys Ala Lys Met Gln Asp Leu Leu Lys Lys Ala Ser Glu
 115 120 125
 Glu Asp His Thr Asn Ala Ala Cys Phe Ala Cys Ile Leu Leu Ser His
 130 135 140
 Gly Glu Glu Asn Val Ile Tyr Gly Lys Asp Gly Val Thr Pro Ile Lys
 145 150 155 160
 Asp Leu Thr Ala His Phe Arg Gly Asp Arg Cys Lys Thr Leu Leu Glu
 165 170 175
 Lys Pro Lys Leu Phe Phe Ile Gln Ala Cys Arg Gly Thr Glu Leu Asp
 180 185 190
 Asp Gly Ile Gln Ala Asp Ser Gly Pro Ile Asn Asp Thr Asp Ala Asn
 195 200 205
 Pro Arg Tyr Lys Ile Pro Val Glu Ala Asp Phe Leu Phe Ala Tyr Ser
 210 215 220
 Thr Val Pro Gly Tyr Tyr Ser Trp Arg Ser Pro Gly Arg Gly Ser Trp
 225 230 235 240
 Phe Val Gln Ala Leu Cys Ser Ile Leu Glu Glu His Gly Lys Asp Leu
 245 250 255

-33-

Glu Ile Met Gln Ile Leu Thr Arg Val Asn Asp Arg Val Ala Arg His
 260 265 270

Phe Glu Ser Gln Ser Asp Asp Pro His Phe His Glu Lys Lys Gln Ile
 275 280 285

Pro Cys Val Val Ser Met Leu Thr Lys Glu Leu Tyr Phe Ser Gln
 290 295 300

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "PCR oligonucleotide primer"
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCATCCTAAT ACGACTCACT ATAGGGC

27

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "PCR primer oligonucleotide"
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCAAACTCTG TCAATTCACC C

21

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 72 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid

-34-

(A) DESCRIPTION: /desc = "PCR oligonucleotide primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCTGCAGTCT AGAGCTCCAT GGCTAGCATG ACTGGTGGAC AGCAAATGGG TGCTAAGCCA 60
GACCGGTCCT CG 72

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PCR oligonucleotide primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GAATTCTAGA TTCTATTGAC TGAAGTAGAG TTC

33

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "site-directed mutagenesis oligonucleotide"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CATTGAGGCT AGCCGAGGGA C

21

(2) INFORMATION FOR SEQ ID NO:8:

-35-

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "site-directed mutagenesis oligonucleotide"
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
GCTATTACTC AGCTGAGAGC CCAGGA

26

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "PCR primer oligonucleotide"
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
CGGCTCGAGG CTAAGCCAGA CCGGTCCTCG

30

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "PCR primer oligonucleotide"
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

(2) INFORMATION FOR SEQ ID NO:11:

- [illegible]

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CLAIMS

We claim:

1. An isolated DNA sequence encoding an active, activatable or inactive CMH-1 polypeptide selected from:
 - a. the DNA sequence set forth in SEQ ID NO:1;
 - b. DNA sequences which hybridize to SEQ ID NO:1; and
 - c. DNA sequences which but for the degeneracy of the genetic code would hybridize to the DNA sequences defined in a. and b. and which sequences code for a polypeptide having the same amino acid sequences.
2. An isolated DNA sequence encoding an active CMH-1 polypeptide selected from:
 - a. a DNA sequence comprising nucleotides 123-962 of SEQ ID NO:1;
 - b. a DNA sequence comprising nucleotides 138-962 of SEQ ID NO:1;
 - c. DNA sequences which hybridizes to the DNA sequences defined in a. or b.; and
 - d. DNA sequences which but for the degeneracy of the genetic code would hybridize to the DNA sequences defined in a., b. or c. and which sequences code for a polypeptide having the same amino acid sequences.
3. The DNA sequence according to claim 2, selected from DNA sequences which encode amino acids 24-303 of SEQ ID NO:2 and DNA sequences which encode amino acids 29-303 of SEQ ID NO:2.

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4. An isolated DNA sequence encoding an inactive CMH-1 polypeptide selected from:
 - a. DNA sequences which hybridize to SEQ ID NO:1; and
 - b. DNA sequences which but for the degeneracy of the genetic code would hybridize to the DNA sequences defined in a. and which sequences code for a polypeptide having the same amino acid sequences; said DNA sequence encoding a polypeptide with no detectable cysteine protease activity.
5. A recombinant DNA molecule comprising a DNA sequence according to claim 1.
6. The recombinant DNA molecule according to claim 5, wherein said DNA sequence is operatively linked to an expression control sequence, said DNA sequence capable of being expressed in a host cell transformed with said recombinant DNA molecule.
7. A prokaryotic or eukaryotic host cell transformed with a recombinant DNA molecule according to claim 5.
8. A prokaryotic or eukaryotic host cell transformed with a recombinant DNA molecule according to claim 6.
9. The host cell according to claim 7 or 8, wherein said host cell is a mammalian cell.
10. The host cell according to claim 7 or 8, wherein said host cell is a E. coli.

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11. A method of producing a DNA sequence encoding an active, activatable or inactive CMH-1 polypeptide comprising the steps of:

- a. incubating a host cell according to claim 7 in a growth medium;
- b. separating said cells from said medium; and
- c. isolating said DNA sequence from said cells.

12. A method for producing an active or activatable CMH-1 polypeptide comprising the steps of:

- a. incubating a host cell according to claim 8 in a growth medium under conditions which promote expression of said ~~CMH-1~~ polypeptide;
- b. separating said cells from said medium; and
- c. isolating said polypeptide from said cells.

13. An active or activatable CMH-1 polypeptide.

14. An antibody to a CMH-1 polypeptide selected from polyclonal antibodies, monoclonal antibodies and antibody fragments capable of binding to a CMH-1 polypeptide.

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Protein	Catalytic	P1	P2	P3	P4
CMH-1 aa #	144 186	87 184 233 239	229 232	180 235	234 237 240 275
CMH-1	H C	R Q R S	Y W	N P	S R W S
CPP32	H C	R Q R S	Y W	S S	N D W S
MCH2	H C	R Q R S	Y H	R T	E N W D
ICH1	H C	R Q R S	A M	S T	N R W Y
ICErelIII	H C	R Q R S	V W	N R	D R I K
TX	H C	R Q R S	V W	N S	D M I K
ICE	H C	R Q R S	V W	T P	H M V R
ICE aa #	237 285	179 283 341 347	338 340	180 343	342 345 348 383

FIG.1

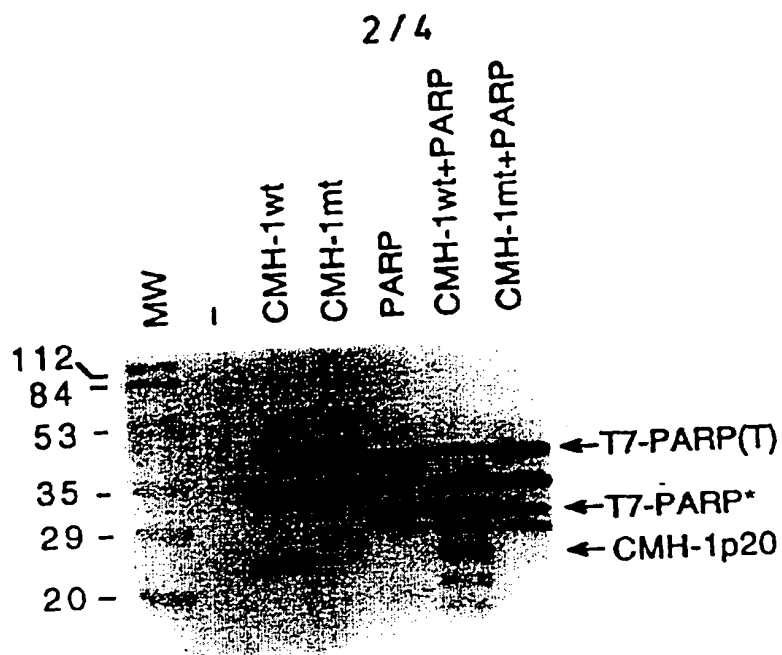


FIG.2A

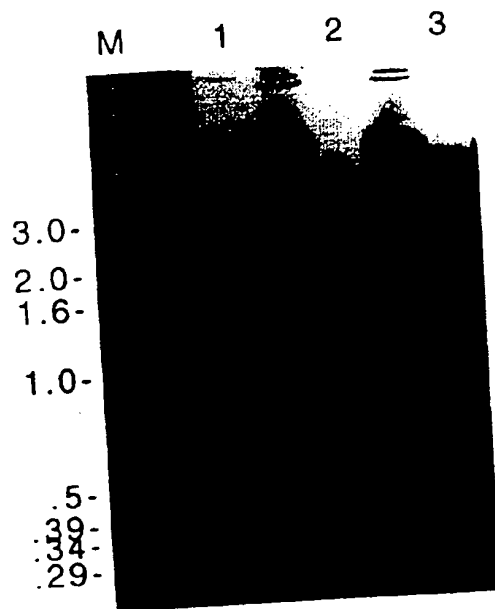


FIG.2B

3 / 4

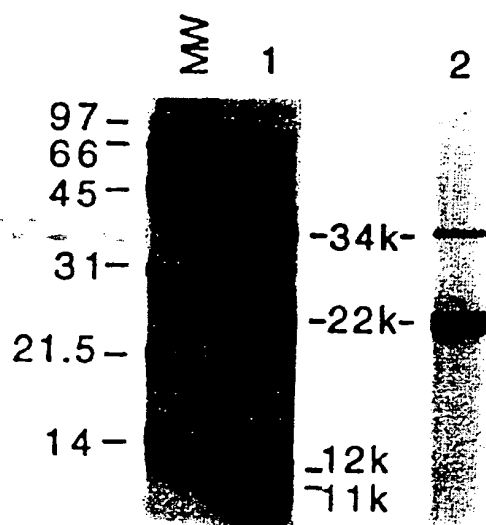


FIG.3

4 / 4

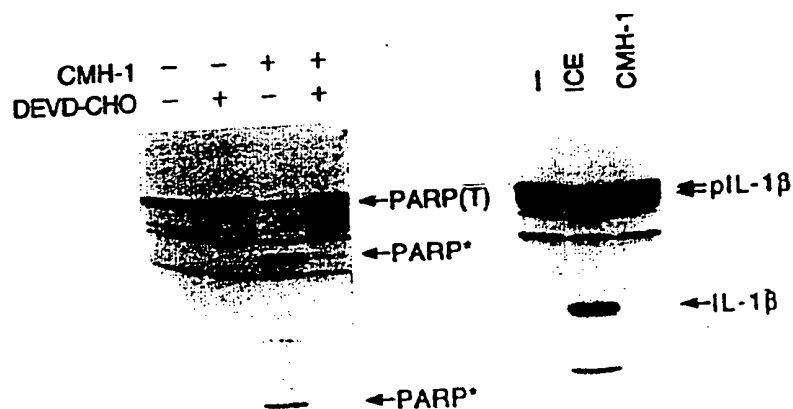


FIG.4A

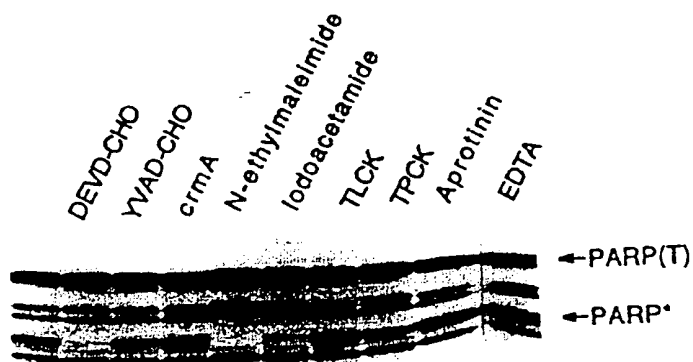


FIG.4B

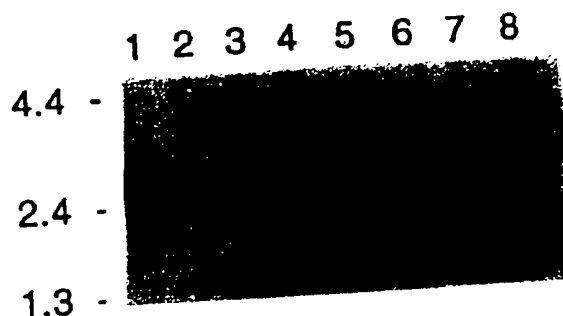


FIG.5

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 96/17431

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/52 C12N9/64 C07K16/40

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>J. BIOL. CHEM., vol. 269, no. 49, 9 December 1994, pages 30761-30764, XP000616157 T. FERNANDES-ALNEMRI ET AL.: "CPP32, a novel human apoptotic protein with homology to Caenorhabditis elegans cell death protein Ced-3 and mammalian Interleukin-1beta-converting enzyme." cited in the application see the whole document</p> <p style="text-align: center;">--- -/-</p>	

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

& document member of the same patent family

Date of the actual completion of the international search

11 February 1997

Date of mailing of the international search report

05.03.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax (+31-70) 340-3016

Authorized officer

Hix, R

INTERNATIONAL SEARCH REPORT

Inter. Appl. No.
PCT/US 96/17431

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>CELL, vol. 81, 2 June 1995, pages 801-809, XP002013933 M. TEWARI ET AL.: "Yama/CPP32beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase." cited in the application see the whole document ---</p>	
A	<p>NATURE, vol. 376, 6 July 1995, pages 37-43, XP000574812 D.W. NICHOLSON ET AL.: "Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis." cited in the application see the whole document ---</p>	
P,X	<p>JOURNAL OF BIOLOGICAL CHEMISTRY 271 (4). 1996. 1825-1828. ISSN: 0021-9258, 26 January 1996, XP000616154 LIPPKE J A ET AL: "Identification and characterization of CPP32- Mch2 homolog 1, a novel cysteine protease similar to CPP32." see the whole document ---</p>	1-14
P,X	<p>CANCER RESEARCH 55 (24). 1995. 6045-6052. ISSN: 0008-5472, 15 December 1995, XP000614740 FERNANDES-ALNEMRI T ET AL: "Mch3, a novel human apoptotic cysteine protease highly related to CPP32." see the whole document ---</p>	1-14
P,X	<p>J. BIOL. CHEM. (1996), 271(3), 1621-5 CODEN: JBCHA3;ISSN: 0021-9258, 19 January 1996, XP000616150 DUAN, HANGJUN ET AL: "ICE - LAP3, a novel mammalian homolog of the Caenorhabditis elegans cell death protein Ced-3 is activated during Fas- and tumor necrosis factor-induced apoptosis" see the whole document ---</p>	1-14

-/--

INTERNATIONAL SEARCH REPORT

International Application No

PC/US 96/17431

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 93 (11). 1996. 5437-5442. ISSN: 0027-8424, 28 May 1996, XP000616168 PAI J-T ET AL: "Purification and cDNA cloning of a second apoptosis-related cysteine protease that cleaves and activates sterol regulatory element binding proteins." see the whole document ---	1-14
P,A	JOURNAL OF BIOLOGICAL CHEMISTRY 271 (18). 1996. 10816-10820. ISSN: 0021-9258, 3 May 1996, XP000616152 GU Y ET AL: "Processing and activation of CMH -1 by granzyme B." see the whole document -----	1-14